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Award Number: DAMD17-00-1-0145

TITLE: Mechanism of Mutation in Non-Dividing Cells

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Annual Summary(1 Jul 01 - 30 Jun 02)	
4. TITLE AND SUBTITLE Mechanism of Mutation in Non-Dividing Cells			5. FUNDING NUMBERS DAMD17-00-1-0145	
6. AUTHOR(S) Rebecca G. Ponder Susan Rosenberg				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, Texas 77030 E-Mail: rp692236@bcm.tmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color			20021230 205	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Stationary-phase mutation is a mutational program that can be induced in non-dividing cells after exposure to environmental stress. We tested the postulate that stationary-phase mutations result from acts of DNA double-strand break repair. In one model for stationary-phase mutation, a DSB intermediate primes DNA synthesis, during which pol IV, an error-prone polymerase required for stationary-phase Lac ⁺ mutation, is proposed to create errors that lead to mutation. F plasmid transfer (Tra) proteins are required for stationary-phase reversion of a +1 frameshift mutation on an F' sex plasmid. Tra functions induce single-strand nicks on the F', which could lead to DSBs. We find that introducing specific breaks on an F' that lacks Tra functions results in 50-2000-fold stimulation of Lac ⁺ stationary-phase mutation. These results provide the first direct evidence that DNA DSBs can activate stationary-phase mutation and imply that the role of Tra functions is to promote the formation of DSBs. We report that DSB-stimulated mutation requires recombination proteins and DNA pol IV. This indicates that introduction of DSBs activates a similar mechanism to that which produces Lac ⁺ stationary-phase mutation, and not an alternative pathway, and that the recombination and polymerase functions are required after DSB formation.				
14. SUBJECT TERMS Breast Cancer, Mutation, Recombination, Error-Prone Polymerase			15. NUMBER OF PAGES 11	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction:

Stationary-phase mutation, or adaptive mutation, refers to a collection of mutagenic responses that can be induced in stationary-phase (non-growing) cells after exposure to environmental stress. In the *E. coli* Lac system, cells carrying a chromosomal *lac* deletion and an F' sex plasmid with a *lac* +1 frameshift allele generate Lac⁺ reversion mutants over time when starved on medium with lactose as the only carbon source. The mechanism for stationary-phase mutation is intrinsically different from that of growth-dependent mutation; it requires the homologous recombination proteins RecA, RecBCD, and RuvA, RuvB, and RuvC. RecA is a homolog of the human protein RAD51, which associates with the DNA repair BRCA tumor suppressor proteins. RecBCD is the major DSB-repair enzyme in *E. coli*. The SOS-inducible, error-prone DNA polymerase, polIV (or DinB) is also required for stationary-phase Lac⁺ reversion; this enzyme is a homolog of four new human DNA polymerases: RAD30a (the XPV tumor suppressor protein), RAD30b, REV1, and DINB1. The mechanisms by which these proteins act in environmentally-inducible mutation are likely relevant to cancer formation, tumor progression, and chemotherapeutic drug resistance in humans.

DNA double-strand breaks (DSBs) have been implicated as molecular intermediates to stationary-phase mutation because of the requirement for RecBCD; the enzyme loads only onto DNA ends. In one model, recombination-mediated repair of a DSB is suggested to promote mutation by priming DNA replication using DNA polIV, during which polymerase errors occur. Cells carrying mutations that revert the *lac* +1 frameshift are able to utilize lactose in the medium and grow, escaping stress. Although stationary-phase mutation appears to occur throughout the genome, on the bacterial chromosome as well as the F' sex plasmid, the frequency of mutation varies widely from locus to locus. For example, the F' *lac* +1 frameshift normally used in our assays mutates at a frequency of about 1×10^{-6} mutants per cell over the course of five days, whereas the frequency of mutation of a frameshift at the chromosomal *lac* locus is less than 1×10^{-8} . We hypothesize that DSBs activate mutation in stationary-phase, and the rate of recombination-dependent mutation at a locus is directly affected by its proximity to DSBs.

DSBs could arise naturally in cells from DNA synthesis across an existing single-stranded nick, an induced enzymatic activity in stationary-phase, or an increased rate of oxidative damage (and its processing by endonucleases during repair). We hypothesize that the chromosomal *lac* locus has a low level of stationary-phase mutation because it lacks sufficient natural DSBs. In the case of the F', we know that plasmid-encoded transfer (Tra) proteins are required for stationary-phase mutation, although actual conjugative transfer is not. An endonuclease called TraI induces single-strand nicks at the origin of transfer on the F', and there are many ways in which a nick might become a DSB, such as a nick on the opposing DNA strand or passage of a replication fork. We hypothesize that Tra proteins activate mutation on the F' because they promote DSBs by providing single-strand nicks. The goal of this project is to determine the role of DNA DSBs and DSB repair in Lac⁺ recombination-dependent stationary-phase mutation in *E. coli*.

Body:

We have asked whether DSBs introduced specifically near *lac* on the F' can 1) activate stationary-phase mutation and 2) substitute for Tra functions. To make specific DSBs, I constructed strains that express the *S. cerevisiae* endonuclease I-SceI (similar to yeast HO endonuclease, as described in SOW Task 1(b), months 1-5) under the arabinose promoter, P_{BAD} , from *attB* in the *E. coli* chromosome. At the same time, I cloned the I-SceI restriction site, an 18bp sequence not present in the *E. coli* genome, into a defective miniTn7, and moved the miniTn7 into multiple sites to the left and to the right of the +1 *lac* frameshift mutation on the F' sex plasmid. Once the desired cut sites were identified, I constructed strains that carry the specific I-SceI restriction sites on an F' deleted for *TraI* endonuclease and either the P_{BAD} -I-SceI gene or P_{BAD} alone at *attB* in the *E. coli* chromosome. These strains were actually constructed twice during the first six months of my fellowship due to a bacteriophage contamination problem we had in the lab. The reconstruction took nearly two months, but the new set of strains has shown no sign of contamination in multiple tests.

P_{BAD} is induced by arabinose and repressed by glucose, so we can control expression of I-SceI in our strains. However, if we plate the cells on arabinose and induce DSBs, death is observed (only) in strains carrying both the I-SceI gene and a cut site. In the stationary-phase mutation assays I am doing, cultures of the strains to be tested are grown in minimal glycerol medium with 0.001% glucose added for repression of the arabinose promoter. Cultures are washed twice and plated on minimal lactose plates without arabinose, so any expression of the I-SceI endonuclease is driven by leaky expression from P_{BAD} in the absence of glucose. The number of Lac^+ colonies are then counted daily until five days after plating. Under these conditions, strains carrying both the I-SceI gene and a cut site still exhibit some death, such that the number of viable cells drops three- to five-fold over the course of five days.

In repeated sets of experiments (SOW Task 1(b), months 10-15), introduction of specific DSBs at two cut sites to the left of the *lac* +1 frameshift on an F' lacking the *TraI* single-strand endonuclease caused dramatic 2000-fold stimulations of Lac^+ stationary-phase mutation (figure 1). This effect was DSB-dependent because no increase in mutation was seen in any of the controls with enzyme but no cut site or cut site but no enzyme. Likewise, introduction of specific DSBs at two cut sites to the right of *lac* activated stationary-phase mutation on a *Tra*-defective F'; one cut site gave a 50-fold stimulation, while the other showed a 1000-fold effect (figure 2). These results provide the first direct evidence that DSBs can activate stationary-phase mutation and imply that the only role of *Tra* functions is to promote DSBs. I previously saw that the frequency of mutation in strains carrying both the I-SceI gene and a cut site was variable from experiment to experiment and culture to culture, but this problem was due to variations in shaking/oxidation of the cultures during growth and has been eliminated.

We have also asked whether the introduction of DSBs activates a similar mechanism to that which produces Lac^+ stationary-phase mutation, requiring recombination proteins and DNA pol IV, or an alternative pathway. In repeated sets of experiments, loss of any of the recombination proteins *RecA*, *RecB*, and *RuvC* resulted in a dramatic decrease of the DSB-stimulated mutation (figure 3). Similarly, the break-promoted mutation required DNA pol IV (figure 3). These results indicate, first, that

introduced DSBs near *lac* activate a mutation mechanism(s) similar to those stationary-phase mechanisms normally observed in the Lac system. Second, these data indicate that the functions of RecA, RecBCD, Ruv proteins, and DinB/Pol IV in stationary-phase mutation are required after DSB formation (DSBs can not substitute for them). This result rules out previously plausible models in which these proteins act solely in generation of DSBs and supports models in which stationary-phase mutation is directly associated with DSD-repair.

We are currently asking whether DSBs can overcome the requirement that cells be non-dividing or slowly-growing to undergo stationary-phase mutation. That is, we are asking whether DSBs can activate a recombination-dependent, DNA pol IV-dependent mutational mechanism(s) in growing cells. We initially attempted to use a Lac assay to measure "growth-dependent" reversion of the same +1 frameshift mutation we normally assay, but found that we could not distinguish Lac⁺ growth-dependent mutants from Lac⁺ stationary-phase mutants that were also coming up under the selection. We are currently asking whether introduction of DSBs in growing cells can increase DNA pol IV-dependent reversion of a *tet* +1 frameshift mutation located near *lac* on the F' sex plasmid; mutants are selected on the antibiotic tetracycline. Preliminary experiments indicate that introduction of DSBs in growing cells does not activate reversion of the *tet* +1 frameshift in comparison to controls. If true, this result would indicate that there is some other component that is provided in stationary-phase cells that is necessary for the mutational mechanism(s) to activate.

We are also currently asking whether DSBs activate mutation by a *cis* or a *trans* mechanism. In the "*cis*" model for stationary-phase mutation I described above, a DSB that occurs near *lac* leads to an SOS DNA damage response and increased levels of DNA pol IV. Repair of the DSB creates recombination intermediates that are proposed to prime error-prone DNA synthesis by DNA pol IV at *lac*. In this model, the initiating DSB, recombinational repair, and resulting mutation all occur in *cis* on the DNA. However, we could also draw a "*trans*" model for stationary-phase mutation which is initiated by a DSB anywhere in the genome. In this model, a DSB leads to induction of the SOS response. Pol IV is upregulated during SOS, and makes polymerase errors in areas of DNA synthesis throughout the cell, in *trans* to the recombinational repair of the initiating DSB. If the *cis* model is correct, and the rate of recombination-dependent mutation at a locus is directly affected by its proximity to DSBs, then we should be able to increase mutation on a Tra-defective F' by inducing specific DSBs in *cis*, but not in *trans*, to the target. If the *trans* model is correct, then specific DSBs induced in *cis* and in *trans* to a target should both promote mutation.

I have already shown that specific DSBs in *cis* to *lac* can activate stationary-phase mutation on a Tra-defective F'. To test whether DSBs made in *trans* would also activate mutation, I created strains that carry either the P_{BAD}-I-SceI gene or P_{BAD} alone at *attB* and an I-SceI cut site at *upp* in the chromosome. In repeated sets of experiments, introduction of specific DSBs at *upp*, in *trans* to the *lac* +1 frameshift on a Tra-defective F', had no effect on Lac⁺ stationary-phase mutation. Unfortunately, we could not conclude from this result that DSBs activate stationary-phase mutation by a *cis* mechanism because we could not show that similar numbers of DSBs were created at the various cut sites on the F' and the chromosome. We also could not control for the possibility that introducing DSBs in the chromosome was more lethal to a cell than making breaks on the F'. To

overcome these difficulties, we decided to assay the effect of DSBs made on a third replicon, a pBR322-based plasmid. In this case, making DSBs would not affect cell viability. In one preliminary experiment, introduction of specific DSBs at a cut site on a plasmid activated stationary-phase mutation in *trans*, on a Tra-defective F', approximately 10-fold. Notably, when homology to the cut site-bearing plasmid is placed near *lac* on the F', the level of mutation is increased an additional 10-fold. These results, if true, indicate 1) that stationary-phase mutation can occur by a *trans* mechanism, if only slightly, and 2) that homologous interactions stimulate stationary-phase mutation (similar to experiments outlined in Task 2, but not quite the same). We may in the future assay for direct exchange of markers between the plasmid and F' to ask whether recombined DNA is linked to DNA that has mutated.

All of the work described thus far has studied activation of stationary-phase mutation on a Tra-defective F'. I have also constructed a similar set of I-SceI strains to ask whether introduction of specific DSBs can activate reversion of a +1 frameshift at the chromosomal *lac* locus, a site notoriously cold for stationary-phase mutation. Experiments using these strains have been placed on hold. Please be aware that none of this material has been published, with the exception of the P_{BAD}-I-SceI allele construction.

Key Research Accomplishments (July 2000-July 2002):

- Gathered the first direct evidence that DSBs activate Lac⁺ stationary-phase mutation
- Demonstrated that DSBs can substitute for transfer functions in stationary-phase mutation
- Showed that the DSB-stimulated mutation requires recombination proteins and DNA pol IV
- Mentored 5 students in projects dealing with mutation and recombination in *E. coli*.

Reportable Outcomes:

Publications:

L. M. Gumbiner-Russo et al. 2001. The TGV Transgenic Vectors for Single Copy Gene Expression from the *Escherichia coli* Chromosome. *Gene* 273: 97-104.

Presentations:

Rebecca G. Ponder and Susan M. Rosenberg. Direct Evidence for Double-Strand Breaks in Stationary-Phase Mutation. 2001 Molecular Genetics of Bacteria and Phages Meeting, Madison, WI.

Conclusions:

The mechanism for stationary-phase mutation requires the homologous recombination proteins RecA, RecBCD, and RuvABC and the SOS-inducible, error-prone DNA polymerase, polIV. Some of these prokaryotic DNA repair and mutation proteins are homologs of human DNA damage response proteins; RecA is a homolog of hRAD51, which associates with the DNA repair BRCA tumor suppressor proteins, and *E. coli* DNA polIV, or DinB, is a homolog of four new human DNA polymerases: RAD30a (the XPV tumor suppressor protein), RAD30b, REV1, and DINB1. The mechanisms by which these proteins act in environmentally-inducible mutation are likely relevant to cancer formation, progression, and resistance to chemotherapeutic drugs in humans.

We find that introducing specific breaks at sites on either side of *lac* on a transfer-defective F' causes 50-2000-fold stimulations of *E. coli* Lac⁺ stationary-phase mutation. This activation of mutation occurs only when both the I-SceI enzyme and cut site are present and requires recombination proteins and DNA pol IV. The data imply that introduction of DSBs can overcome the requirement for Tra functions, and provide direct evidence that DSBs can activate stationary-phase mutation in the Lac system. Ongoing work in the lab is addressing whether DSBs can activate DNA pol IV-dependent mutation in growing cells and whether DSBs activate mutation by a *cis* or a *trans* mechanism.

Figure 1

DSBs to the left of *lac* ↑↑ mutation

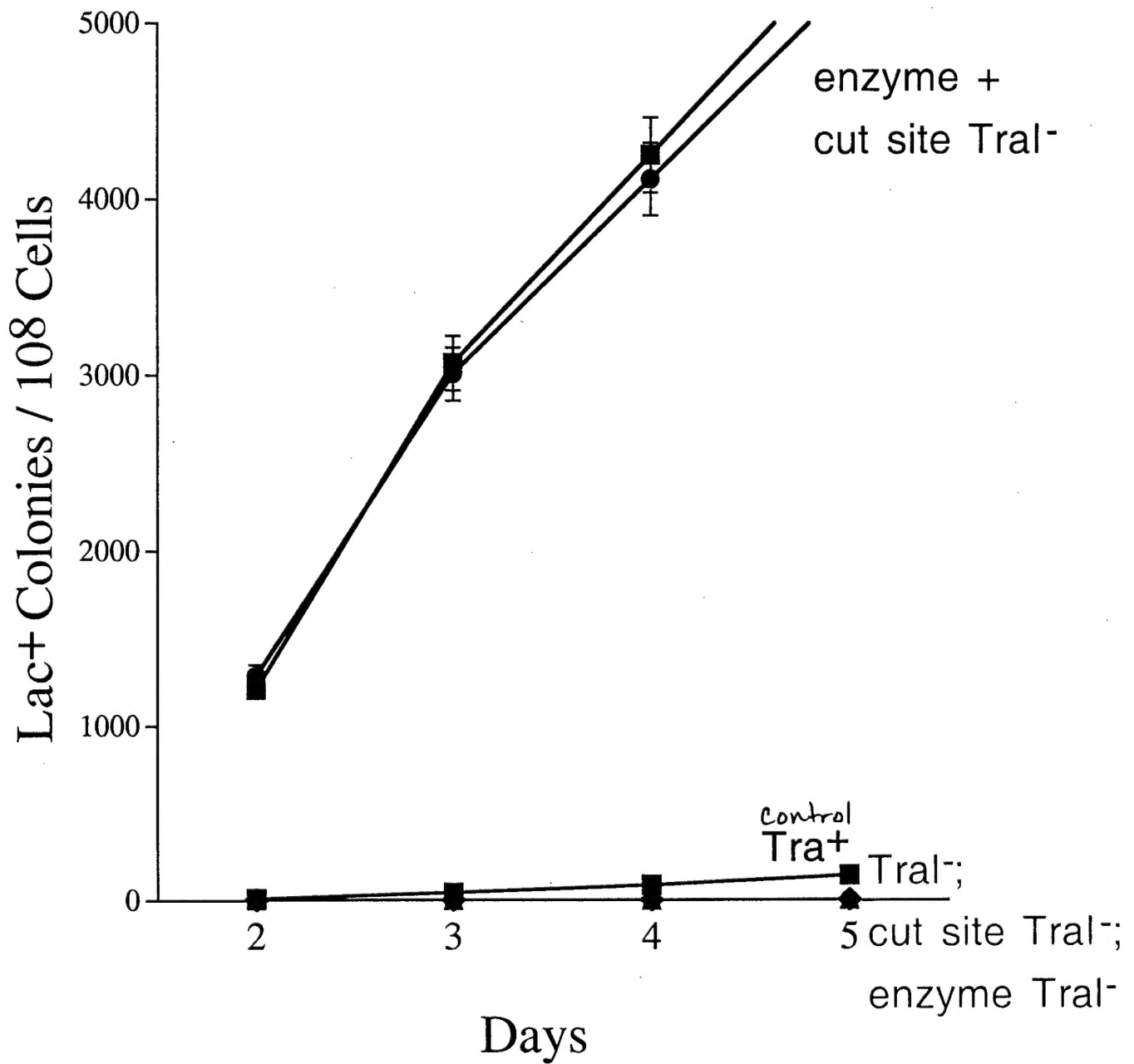


Figure 2

DSBs to the right of *lac*↑↑ mutation

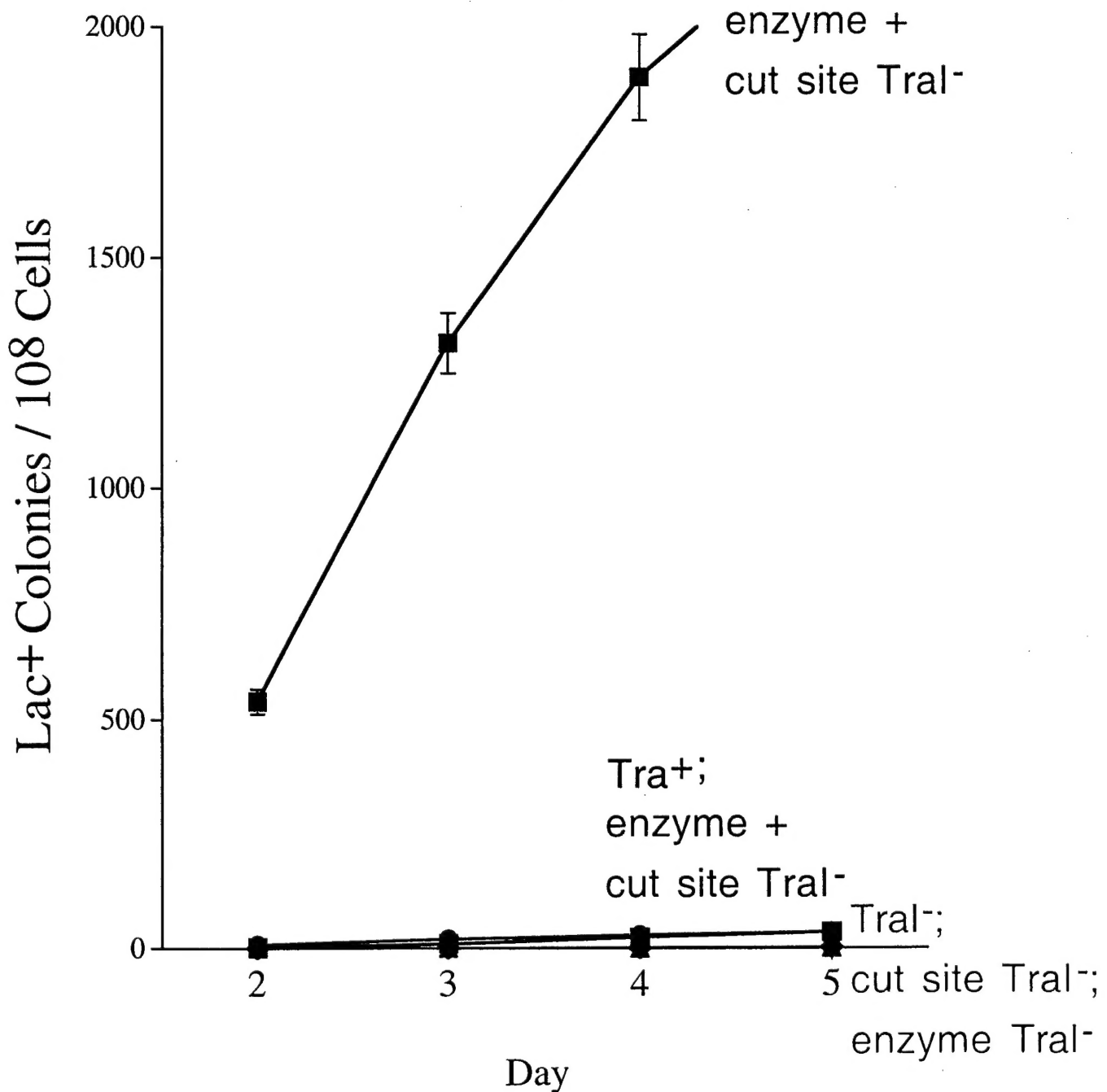


Figure 3

DSB-induced mutation requires
Rec proteins and DNA polIV

